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ORIGINAL RESEARCH

Molecular Investigations of Linezolid Resistance in Enterococci *OptrA* Variants from a Hospital in Shanghai

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Purpose: The OptrA protein is a member of ATP-binding cassette (ABC) transporters, a transporter family which can confer resistance to oxazolidinone antibiotics by transferring plasmid. We aim to describe the distribution of *optrA*-harbored *Enterococcus* in Huashan hospital in 2017 and to address the effects of *optrA* mutations on the susceptibility of linezolid antibiotic drug.

Methods: Linezolid-resistance-related genes were tested for *Enterococcus* by polymerase chain reaction (PCR) and then sequenced for amino acid substitution site analysis. Broth microdilution and agar dilution test were applied to determine the minimal inhibitory concentration (MIC) of linezolid for *Enterococcus* containing *optrA*. Pulsed field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) were used to evaluate the genotypes of *optrA*-positive isolates. To verify the functions of two main *optrA* variants, *optrA* over-expressing strains were constructed.

Results: Among 20 *optrA*-positive strains, only two were resistant to linezolid. No amino acid substitution existed in 23S rRNA V domain among *Enterococcus faecalis*. None had *cfr, cfr*(B) or *cfr*(C) genes. F101L and G4D/K/R or T150A were the main substitutions of ribosomal protein L4, L3, respectively. We found one *Enterococcus faecium* isolate co-contained *optrA* and *poxtA* and another *E. faecalis* isolate co-contained *optrA* and *cfr*(D), but they were not resistant to linezolid. Among 20 *optrA*-positive strains, ST-16 was the main type. Two main *optrA* variants KD (T112K, Y176D) and RDK (I104R, Y176D, E256K) slightly raised enterococci's MIC of linezolid.

Conclusion: OptrA exists in linezolid non-resistant enterococci with diverse amino acid substitutions. The variants play different roles in changing the MIC of linezolid.

Keywords: linezolid, resistant, enterococci, *optrA*

Introduction

Enterococci are medically important opportunistic pathogen which are more common in hospital-acquired infections. Linezolid[1] is the first clinically used oxazolidinone antibiotic for the treatment of multidrug-resistant (MDR) gram-positive infections, especially in vancomycin-resistant enterococci (VRE) and methicillin-resistant *Staphylococcus aureus* (MRSA).[2] However, linezolid-resistant enterococci have been increasingly detected both in human and animals[3] in recent years, which urgently demands to deepen our knowledge on its resistance mechanisms.

The resistance of linezolid in enterococci can be caused by point substitutions in 23S rRNA V domain, the main reason for linezolid resistance.[4] The 23S rRNA

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methyltransferase coding genes *cfr*,[4] *cfr*(B), *cfr*(C)[5] and *cfr*(D)[6] and also amino acid substitutions in ribosomal proteins L3, L4,[4] L22,[7] and ABC-F family members including OptrA[8] and PoxtA.[9] How ABC-F proteins mediate antibiotic resistance has been in a long-standing dispute with both efflux and ribosome protection hypothesis competing mutually. Current studies seem inclined to the latter.[10,11] The *optrA*, found in 2015, was reported for its ATP hydrolysis activity to be associated with linezolid resistance.[12] Also, previous study reported that there were different amino acid substitutions in *optrA*.[13] In this study, we investigated the molecular characterization of *optrA*-positive enterococci and explored the function of varied types of *optrA* on the linezolid susceptibility.

Materials and Methods

Bacterial Strains

We examined 573 strains collected in 2017 at Huashan hospital, a tertiary hospital from Shanghai in China (Table 1). All were enterococci identified by bioMerieux Vitek-2 Compact and were stored in 30% glycerol broth under −80°C. The culture medium used in this study were BHIA (Brain-Heart Infusion Agar), BHIB (BrainHeart Infusion Broth), MHA (Mullex-Hinton Agar) and MHB (Mullex-Hinton Broth) (Thermo Fisher Oxoid, England).

Screening of *optrA* and Other Linezolid Resistance Related Genes in Enterococci

The length of *optrA* is 1968 bp.[8] We screened 573 enterococci mentioned above by using PCR. All DNA samples were amplified using primers *optrA*-F1 (5′-CAGGT GGTCAGCGAACTAAGA-3′) and *optrA*-R1 (5′-AGCC AAGAGCAGTTCTGACC-3′), of which the product size was 792 bp. The thermal conditions were 1 cycle of 94°C for 5 min followed by 30 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 30 s and 72°C for 5 min to stop the extension step. Then, amplifications were carried out in the *optrA*-positive strains for getting the whole sequence of *optrA* and detecting the existence of *cfr*,[14] *cfr*(B), *cfr*(C), *poxtA* and mutations in *rplC, rplD*,[15] *rplV*, and 23S rRNA V domain[16] which were associated with linezolid resistance. Primers of these amplifications were shown in Supplementary 1.

Table 1 The Distribution of Different Species of Enterococcus Isolates from Huashan Hospital in 2017

Bacterial Strains	Number (n)	Percentage
E. faecalis	296	51.66%
E. faecium	264	46.07%
E. avium	9	1.57%
E. casseliflavus	2	0.35%
E. gallinarum	2	0.35%
Total	573	100.00%

Antimicrobial Susceptibility Testing

The MIC for all *optrA*-positive isolates were determined by broth microdilution and agar dilution tests following the recommendations given in the Clinical and Laboratory Standards Institute (CLSI) documents M100-ED28.[17] *Enterococcus faecalis* ATCC29212 served as the quality control strain. These antimicrobial susceptibility tests involved 18 antibiotics (Shanghai Food and Drug Administration, Shanghai, China) which were listed in Supplementary 4. All tests were performed by using broth microdilution except for fosfomycin which was practiced with agar dilution. In CLSI, the approved MIC testing of fosfomycin is agar dilution, and the agar media should be supplemented with 25 μg/mL of glucose-6-phosphate.

Molecular Typing of *optrA*-Positive Enterococci

The phylogenetic distance among isolates harboring *optrA* was analyzed by PFGE. The sequence typing (ST) of all *optrA*-positive enterococci were identified through MLST. The MLST analysis was on the basis of amplifying the seven housekeeping genes *gdh, gyd, psts, gki, aroE, xpt, yqil* in *E. faecalis* and the other seven genes *atpa, ddl, gdh, purk, gyd, psts, adk* in *Enterococcus faecium*. According to the sequences provided in PubMLST (https://pubmlst.org/data/), primers for MLST were designed. All STs were determined by using the *Enterococcus* MLST database (https://pubmlst.org/efaecalis/and https://pubmlst.org/efaecium/).

Determination on the Function of Various Types of *optrA*

From the study published in 2015,[8] the *optrA* found in plasmid pE394 (GenBank accession no. KP399637.1) was defined as the wildtype. A plasmid called *E. faecalis* JH2-2_pAM401Ω*optrA* was constructed to confirm that the *optrA* could result in the resistance of linezolid in enterococci. Therefore, in our research, in order to investigate the effects of the amino acid substitutions of *optrA* on linezolid resistance, we followed the steps in the aforementioned article for the construction of the plasmid

OG1RF_pABG5.0Ω*optrA* (wildtype and mutations). pABG5.0[18] served as the shuttle vector which could exist both in *Escherichia coli* and *E. faecalis*.

In-Fusion HD Cloning Kits (Takara) were used in the experiment. After the successful construction, the plasmid was transferred into OG1RF,[19] a linezolid susceptible *E. faecalis* (MIC=2μg/mL), and then the *optrA*-positive clones were selected for further antimicrobial susceptibility test.

Results

The Distribution of *optrA* and Its Molecular Types

In all 573 strains isolated from Huashan hospital in 2017, we detected 20 *optrA*-positive isolates, numbered as 8, 37, 91, 154, 155, 185, 216, 223, 280, 297, 366, 397, 402, 404, 413, 421, 422, 446, 550, and 551, which accounted for 3.48% of the total strains. In Table 2, we could see that there were 14 *optrA* variants with 1 to 6 alterations at different positions. I104R, T112K, Y176D, and E256K were the most common amino acid substitutions; 18 of 20 strains were separated into 14 various STs, and the remaining two strains could not be identified in known STs.

The detection and amino acid substitutions of other linezolid-resistant-related genes were presented in Supplementary 2. All genes amplified above were compared with the sequences from *E. faecalis* and *E. faecium* attainable in NCBI. (https://www.ncbi.nlm.nih.gov/nuccore/NC_01-7316.1 and https://www.ncbi.nlm.nih.gov/genome/?term=Enterococcus+faecium+DO). None amino acid substitution of 23S rRNA V domain was detected in *E.* faecalis. None of these 20 strains contained *cfr, cfr*(B), *cfr*(C) genes while 397 harbored *cfr*(D) gene. Nineteen strains had F101L mutations of L4, while no mutation of L22 was found, and G4K/D/R were the main mutation sites of L3. Only one strain possessed both *optrA* and *poxtA*.

For further study on whether there was the dissemination of *optrA* in Huashan hospital, we performed PFGE and MLST. Similarity among 20 strain patterns was figured out by computerized band analysis with Bionumerics software version 3.3. This part of results could be seen in Supplementary 3. No obvious homology was found in these 20 strains harboring *optrA*.

Antimicrobial Susceptibility Testing

The results of the antimicrobial susceptibility testing were shown in Supplementary 4. All strains were resistant to

Table 2 Twenty *optrA*-Positive Strains Were Numbered and Their MLST Types Were Listed as Shown Below. We Found the Existence of Various *optrA* Mutations Among Those 20 Which Presented Diverse MICs of Linezolid. According to CLSI, The Criteria of Judging Susceptibility Included Susceptible (MIC≤2μg/mL), Intermediate (MIC=4μg/mL), Resistant (MIC≥8μg/mL)

Isolates	Species	*optrA* Variant	*optrA*	ST	MIC (μg/mL)
8	efa	KD	T112K, Y176D	330	8
37	efa	RDK	I104R, Y176D, E256K	689	4
91	efa	optrAE394	No mutations	476	2
154	efm	D	G40D	362	1
155	efa	optrAE394	No mutations	134	4
185	efa	RDKC	I104R, Y176D, E256K, S399C	376	4
216	efa	KD	T112K, Y176D	460	2
223	efa	DM	Y176D, I622M	16	1
280	efa	KV	T112K, F642V	16	1
297	efa	optrAE394	No mutations	4	4
366	efa	DP	Y176D, T481P	69	4
397	efa	optrAE394	No mutations	476	2
402	efm	DD	Y176D, G393D	/	2
404	efa	KHRDIP	T157K, Y158H, K160R, Y176D, V249I, T481P	69	2
413	efm	NDM	D247N, G393D, I622M	323	2
421	efm	DNDM	Y176D, D247N, G393D, I622M	557	≤0.5
422	efm	K	T112K	78	2
446	efa	RK	I104R, E256K	/	4
550	efa	RDK	I104R, Y176D, E256K	16	2
551	efa	N	I24N	585	8

Notes: K: Lys, D: Asp, R: Arg, C: Cys; M: Met, T: Thr; V: Val, E: Glu, H: His; I: Ile, P: Pro; N: Asn.

tetracycline, kanamycin, erythromycin, and ciprofloxacin while they were susceptible to vancomycin and teicoplanin. The distribution of resistance in the remaining 8 antibiotics were followed by ampicillin (5/20, 25%), penicillin (5/20, 25%), linezolid (2/20, 10%), rifampicin (5/20, 20%), chloramphenicol (15/20, 75%), and fosfomycin (3/20, 15%). Only one strain was found with tigecycline MIC as 4 μg/mL. The imipenem MIC of 4 strains were equal to or over 8 μg/mL. More detailed tests on linezolid were further put into practice. Only two strains were found as linezolid resistant, obtained by broth microdilution separately.

Function of Two Types of *optrA* Variants in Linezolid Resistance

Isolates 8 (*optrA*-KD) and 37 (*optrA*-RDK) were found containing the most common alterations of *optrA* among these 20 strains. Based on this result, they were chosen for the transformation. The successful transformants (designated OG1RF_pABG5.0Ω*optrA*-KD and OG1RF_pABG5.0Ω*optrA*-RDK) exhibited 2 to 4 folds increase in the MIC of linezolid compared with the recipient strain OG1RF (Table 3).

Discussion

In 2017, *E. faecalis* and *E. faecium* were two main types of bacterial pathogens isolated from Huashan hospital. The detection rate of *optrA* in 573 strains was not high. ST-16 was the type that commonly existed among 14 STs (3/20) as mentioned in Table 2 which coincided with the common type of enterococci harboring *optrA* in other districts.[20,21]

Table 3 MICs for Clinical *E. faecalis* with *optrA* Variants, Their Transformants and Recipient Strains

Bacterial Isolates	Linezolid
	MIC/μg/mL
Clinical *E. faecalis*	
HS0914 (wildtype)	8
8 (KD)	8
37 (RDK)	4
Recipient strain	
OG1RF	2
Transformants OG1RF_pABG5.0	2
Transformants OG1RF_pABG5.0Ω*optrA* (wildtype)	8
Transformants OG1RF_pABG5.0Ω*optrA* (KD)	4
Transformants OG1RF_pABG5.0Ω*optrA* (RDK)	4

Notes: HS0914: *optrA* with no mutation; KD: T112K, Y176D type; RDK: I104R, Y176D, E256K type.

These comparisons indicated that *optrA* was carried by low-homology enterococci isolates.

The results of the antimicrobial susceptibility tests have revealed that multidrug-resistant enterococci were not the minority. Resistance levels of linezolid in enterococci containing *optrA* were unevenly distributed.

Through the method of broth microdilution, only two strains were detected with low linezolid resistant level (MIC=8 μg/mL). This conceded that *optrA* was not only present in linezolid-resistant enterococci. At the same time, 20 strains harboring *optrA* had different numbers of amino acid substitutions. In the preliminary experiments, the successful construction of *optrA* over-expressing strains had shown that KD (T112K, Y176D) (BankIt2317497 KD MT122998) and RDK (I104R, Y176D, E256K) (BankIt2317550 RDK MT122999) *optrA* variants were able to raise linezolid MIC but could not reach the MIC as shown in the wildtype *optrA* in pE349. Potential mechanisms of mutations that affected the level of resistance of the bacteria to linezolid deserve further study. However, not all types of variants were used to construct the plasmid for over-expressing the *optrA*. Interestingly, 91 and 397 with no mutation of *optrA* were susceptible to linezolid (MIC=2 μg/mL). Whether *optrA* gene expressed and translated or there were some other mechanisms antagonizing the ribosome protection mechanism which led to the loss of drug resistance phenotype will be another avenue of research.

Screening of other linezolid-resistant genes showed that the *cfr, cfr*(B), and *cfr*(C) detection rates were zero, which were consistent to the results of MIC. Only one *E. faecalis* was detected to carry *cfr*(D).In this year, a study from europe characterized the novel *cfr*(D) gene identified in an *E. faecium* clinical isolate.[6] According to the results mentioned above, it might probably that the *cfr*(D) gene tended to transfer between two kinds of *Enterococcus*. Whether it played a role of conferring linezolid resistance in *Enterococcus* still needs to be explored.

F101L mutation of L3 protein was common among 19 strains. Whether it participated in the process of linezolid resistance remains to be confirmed. For lack of the mature techniques of gene knockout in *Enterococcus*, it is not easy to verify the concrete functions of these substitutions.

The newly discovered PoxtA, which also belongs to ABC-F proteins, shares 32% homology identity with OptrA. Of the 20 strains, only 402 had OptrA (Y176D, G393D) and PoxtA (no alterations). Surprisingly these two displayed no synergistic effects because its MIC showed that it was a susceptible

one. Whether they existed in one plasmid, like pE035 obtained from swine carrying both *optrA* and *poxtA*,[22] or neither was expressed or had other negative interactions is vague at the moment. Moreover, since 421 was extremely susceptible to linezolid (MIC<0.5 μg/mL), whether its regulatory region was interfered still needs to be checked.

In conclusion, although the detection rate of *optrA* in *Enterococcus* spp. isolates was not high, and there was no obvious evidence of its dissemination in hospital, its role in linezolid resistance still needs to be noted. *OptrA* mutations affected the linezolid resistance at varying degrees. Detailed mechanisms such as how *optrA* variants interact with linezolid are of more need to be clarified.

Ethical Statement

The strains we used in this study were obtained from the biological sample and strains bank of the Institute of Antibiotics, Huashan Hospital, Shanghai, China. They came from the normal clinical testing and were stored in the strains bank. The ethics committee of Huashan Hospital authorized our study and written informed consent is not required. This study would not do harm to rights, benefits, and health of the subjects, and the privacy and personal identity information of the subjects will not be included in this study.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest for this work.

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